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Development of a Novel Salivary Cortisol Laboratory Experiment for Undergraduate Physiology Students

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by

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ABSTRACT

Development of a Salivary Cortisol Laboratory Experiment for Undergraduate Physiology Students (Under the direction of Carol A. Britson)

Developing an endocrine-based laboratory exercise for undergraduate students is challenging because of the limitations that are characteristic of endocrine processes. These laboratory exercises often involve invasive methods and expensive biochemical analysis. They are also time consuming because of the slow-acting endocrine responses. A potential endocrine-based exercise that can be completed in 2-3 hours, is non-invasive, and at a low cost is a salivary assay. This assay is not without drawbacks, however, including sample collection outside the laboratory classroom. Cortisol is a hormone that is accessible in saliva and provides some insight into the Hypothalamic-Pituitary-Adrenal Axis (HPA Axis), also known as the stress cascade. Stress has been shown to evoke cravings for foods high in sugar content. Cortisol is often released in response to a stressor and tends to follow a circadian rhythm, tending to be higher in the morning and lower at night. Validation of the procedure for evaluating this circadian rhythm in an undergraduate laboratory setting was achieved by allowing 16 students to provide night and morning saliva samples by self-collecting samples at home. The other 84 students were split into groups to examine the effect of sugar intake on cortisol levels by eating snacks with varying amounts of sugar. These sugar treatment groups consisted of a control group (0g), low (<1g), medium (14g), and high (26g) sugar consumption. Samples were analyzed with a salivary cortisol ELISA assay kit procedure and with a microplate reader to obtain absorbance measurements. A paired t-test supported

significance ($p<0.001$) between morning and night samples of cortisol. ANCOVA was used to determine a significance ($p<0.001$) found between samples in all sugar treatment groups. Therefore, sugar was shown to have an effect of increasing cortisol levels. The methods were successful in the ability to be integrated into an undergraduate laboratory curriculum by limiting the time and invasiveness of an endocrine-based laboratory approach to teaching students.

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INTRODUCTION

There are many physiological responses to stress, with one of the most notable being the Hypothalamic-Pituitary-Adrenal Axis (HPA Axis) response. Effects of the HPA axis include hypothalamic corticotropin-releasing hormone and pituitary adrenocorticotropic hormone release (Haussmann et al., 2006). This response also induces adrenal glucocorticoid release, which consists of increased amounts of cortisol and corticosteroid hormones being found in the body (Haussmann et al., 2006). It can be deduced that measuring the amounts of one of these HPA axis hormones in response to a change in the local environment can provide indirect evidence of the individual feeling stress. This response could have the potential to be assessed in the development of an endocrine-based lab procedure for undergraduate students enrolled in physiology.

The HPA axis can be thought of as a stress cascade, which is useful in times of necessity but is also often induced in response to everyday situations such as during school exams and class or work presentations among other routine events (Haussmann et al., 2006). This is an issue because the body may attribute non life-threatening situations to stress, which would increase the frequency of the stress cascade. This is potentially concerning when considering students who may feel the effects of the stress cascade daily and overreact physiologically.

Stress is also tied to eating behavior, making some people over eat and others under eat. Specifically, foods high in fat and sugar are usually chosen as a reward and may become addictive (Duarte et al., 2014). Feeling stressed leads to a desire to eat more nutrient poor foods that taste better in an attempt to combat the negative feelings

associated with stress. College students are especially prone to changing eating habits because they are usually away from home and are living in a new environment. These changes combine with the stress felt from the desire to perform well in classes, become involved in extracurricular activities, and maintain a social life that leads to erratic and varying eating behaviors among college students (Ramler et.al, 2015; Sladek and Doane, 2014; Stephens et al., 2015; Stowell et al., 2008). Foods high in salt, fat, and sugar are staples of many restaurants aimed toward college students and specifically located on college campuses. The stress felt by students who participate in late night binge studying likely leads to more unhealthy choices being consumed at odd times in the sleep/wake cycle as an incentive or as a reward, which causes hormonal imbalances and disrupts homeostasis (Duarte et al., 2014).

Cortisol levels tend to rise and decline depending on the time of day tending to be higher in the morning and lower in the evenings (Adams and Kumari, 2009; Liu and Vagula, 2015; Sharpley et al., 2010). The cortisol released into the bloodstream can be found in blood, saliva, and urine samples (Sharpley et al., 2010). Cortisol was dispersed from the bloodstream to these other spaces of the body, which contain water (Gozansky et al. 2005). Many hormones, including blood plasma cortisol, tend to be difficult to collect in a non-medical setting due to limited resources and invasive methods needed to collect blood samples; however, salivary cortisol is more accessible, less invasive, and has comparable results to using serum from blood samples (Contreras et al., 2004; Crewther et al., 2010; Gozansky et al. 2005; Tunn et al., 1992; Contreras et al., 2004). Therefore, saliva samples make a viable source to examine this hormone when considering changes in the stress cascade. Liu and Vagula (2015) used a simple assay kit to confirm the

circadian rhythm of cortisol levels by analyzing saliva samples of undergraduate students collected both before and after sleep. There was a significant difference between morning and evening cortisol levels, with the morning levels being higher than the evening levels (Liu and Vagula, 2015).

The Haussmann et al. experiment paired physiological and psychological stressors in the form of graded presentations, competitive games, and fasting to assess the effect of stress on cortisol. Undergraduate students provided samples both before and after participating in one of the stressful activities (Haussmann et al., 2006). Cortisol levels were higher for most people following participation in the stressful activities; however, it is notable to mention that some people actually had lower cortisol levels if they found the activity to be relaxing or enjoyable (Haussmann et al., 2006). The different reactions that people have in doing activities such as presenting and playing competitive games is undesirable in an undergraduate laboratory exercise because it could lead to a greater likelihood of misinterpretation of data (Haussmann et al., 2006). Using fasting as a stressor would also rely too much on individual efforts outside of the laboratory time (Haussmann et al., 2006).

Sugar intake is a physiological stressor that has been studied in monkeys and has the potential to be better suited in an undergraduate laboratory setting than other methods (Duarte et al., 2014). Sugar intake would provide more similar results and be better regulated in undergraduate students than inducing stress or letting students self-collect samples at home. A cortisol experiment that measured changes in cortisol levels for Marmoset monkeys found that sugar affected the behavior of the monkeys but did not affect their blood cortisol levels (Duarte et al., 2014). The Marmoset monkeys also chose

to eat chocolate chips more than the more nutritious option of chow when presented with both (Duarte et al., 2014). Brain scans showed that the foods high in fat, salt, and sugar such as chocolate chips brought more pleasure to the monkeys and became an addiction over time (Duarte et al., 2014). The monkeys even showed signs of anxiety when chocolate chips were not available by searching frantically for them (Duarte et al., 2014). Results revealed that cortisol levels were not influenced by food choice but behavior was and that more chocolate was consumed on average rather than the healthier alternative of chow (Duarte et al., 2014).

The influence of the natural diurnal rhythm of cortisol was accounted for by collecting the blood samples in the same range of time for each collection (Duarte et al., 2014). Cortisol levels were possibly not affected by sugar consumption for the marmoset monkeys of the experiment examining addiction, cortisol, and food choice because the monkeys had not been previously exposed to different foods with varying amounts of sugar or evident stressors relating to food choice.

If differing concentrations of sugar in food consumed causes changes in cortisol levels, then the hectic and stressful schedule of some college students may synergistically encourage eating behaviors that cause cortisol levels to deviate from its natural circadian rhythmicity. Varying the amount of sugar consumed in humans may confirm that although the circadian rhythm plays a larger role in overall cortisol levels by limiting the fluctuation that can be caused by changes in the local environment, interference or minor changes to the internal environment such as is experienced with food choice may also cause transient changes. Since changes in eating behavior are often associated with feelings of stress, it may be considered whether sugar-laden foods chosen to combat

negative feelings of stress affect cortisol levels independently of an obvious stressor and also if this response is momentarily stronger than the circadian rhythm's homeostatic nature (Duarte et al., 2014; Haussmann et al., 2006; Liu and Vagula, 2015). A momentary change could become a larger issue if food high in sugar content is consumed regularly throughout the day, constantly eliciting the HPA axis. Additional elements to the inquiry of sugar intake affecting cortisol release is to consider if different amounts of sugar content in a food item causes an incremental relationship of increasing sugar also resulting in increasing amounts of cortisol being released.

Developing a manageable endocrine-based activity that is engaging for physiology students presents the challenge of finding a treatment that would both demonstrate the process and be of interest to students. Endocrine-based laboratory exercises create a challenge because many elements of the endocrine system involve invasive methods or require a larger portion of time to complete than is available. It is important to find a novel method of relating potential physiological stressors to induce changes in cortisol levels by using a variable that is both accessible and manageable in an undergraduate laboratory setting. Sugar provides an interesting treatment as it is easily accessible, relevant to undergraduate students, and is often related to stress. Using different amounts of sugar content could also potentially relate food choice to endocrine processes by showing that sugar intake may increase cortisol levels. It could also present the importance of macronutrient intake and food quality (Gyllenhammer et al., 2014).

With a sugar treatment method, this laboratory exercise should avoid some of the challenges faced in other endocrine-based undergraduate laboratory exercises. Liu and Vagula's (2015) protocol for an undergraduate laboratory experiment required a

significant commitment by students to collect their own saliva samples at home and follow methods for the safekeeping of the sample before remembering to bring the samples back to lab. Liu and Vagula (2015) serves as a foundation for this experiment because it effectively confirmed the well-established circadian rhythm of cortisol in an undergraduate laboratory setting which I hope to achieve with the added aim of limiting the experiment to a 3-hour lab session and the additional element of including sugar treatment groups.

I hypothesize that a spike in cortisol arises near the time of consumption of nutrient poor foods. This will be accomplished by testing short-term changes in cortisol rather than longer-term changes in cortisol levels. The circadian rhythmicity of cortisol test used by Liu and Vagula (2015) must be validated with the before bed nighttime saliva samples containing lower concentrations of cortisol than the samples upon waking in the morning because of the variability of data collection. I further hypothesize that sugar treatment groups will all experience increased concentrations of cortisol in the post-consumption sample as compared to the pre-consumption sample, and higher amounts of sugar content in each snack will be associated with higher concentrations of cortisol as compared to the sugar-free snack. Sugar should have the effect of activating the HPA axis, resulting in greater release of cortisol (Haussmann et al., 2006). This could support the possibility that sugar acts as a stressor independently of obvious external stressors.

MATERIALS AND METHODS

One hundred undergraduate students enrolled in BISC 206: Human Anatomy & Physiology I at the University of Mississippi volunteered to participate by providing saliva samples during September, October, or November of the Fall 2017 semester. This experiment was IRB approved (protocol # 18-010) and followed guidelines for the safekeeping of collected samples and for the proper treatment of participants. Each student was asked to read and sign a consent form before beginning the collection process. The consent form included a description of the project along with expectations on behalf of the participant. Each student also confirmed that they were over eighteen years of age by filling in a box on the consent form and through verbal confirmation. Of these 100 undergraduate volunteers, 81 students were female and 19 were male. Students were offered extra credit for the BISC 206 class in return for their participation. An alternative assignment was made available for students who were unable to meet at the saliva collection group times and for those who preferred not to provide a sample. 66 students opted to do this alternative assignment instead, which consisted of watching a video about cortisol and the endocrine system and then taking a quiz made up of questions based on that video (Appendix A). The video discussed the circadian rhythmicity of cortisol, diseases associated with cortisol, testing methods for it, situations that cause cortisol to rise, and stress.

Cohort					
	Circadian	Zero Sugar	Low Sugar	Medium Sugar	High Sugar
# of Students	16	19	20	20	25

Table 1: This represents the five different groups for the experiment and the number of undergraduate students in each of these groups (n=100).

There were five total experimental groups consisting of a circadian validation group, a zero sugar control, a low sugar content group, a medium sugar content group, and a high sugar content group. Saliva collection instructions were given verbally to students. Students were asked to let the saliva pool in their mouths before drooling slowly into the tube and were advised to avoid forcefully spitting into the tube. This method ensured that a usable saliva sample was obtained rather than a tube consisting of mostly bubbles. The few students who struggled to produce saliva were told to imagine his or her favorite food in an attempt to illicit saliva production.

The circadian validation group participated on Thursdays by picking up materials consisting of 2 mL micro centrifuge tubes, to hear and receive emailed home collection instructions, and to sign the consent form. Participants in the circadian validation group were asked to freeze the tubes until they could bring the two tubes, with the now collected saliva samples back the afternoon of the next day. Friday afternoons were also scheduled for meeting with the sugar treatment groups. This same schedule was kept constant for multiple weeks throughout the fall semester as students continuously signed up for the date that best fit his or her individual schedule. Each Friday had two groups, which met 30 minutes apart, and around six students signed up for each group time.

Students in the circadian validation group collected a saliva sample in a tube before bedtime that night and without eating anything at least 30 minutes before the time of collection. In the morning immediately upon waking, they collected a saliva sample in the second tube also without eating for at least 30 minutes before the time of collection to avoid extraneous variables such as interference from the temperature or content of food consumed. This was done to help ensure the validity of the data and to avoid obtaining results affected by food intake.

Snacks for the sugar intake treatment groups were based on the sugar content of each snack in one serving size. The sugar groups were based on the percentage of sugar content in the provided snack as related to the percentage of daily value written on the nutrition label. The no sugar control group chewed one stick of sugar-free Orbit® gum, which contained 0g of sugar. The low sugar group consumed a serving of fifteen Lay's® original potato chips, which had less than 1 gram of sugar. The medium sugar content group consumed three Nabisco's Oreo® chocolate sandwich cookies, which contained 14 grams of sugar in one serving. The high sugar content group consumed one serving size of sixteen pieces Sour Patch Kids® candy, which contained 26 grams of sugar.

The sequence of events during group collections for the food groups consisted of having students sign consent forms before providing the first saliva sample in the 2 mL tube. Students were then asked to eat one serving size of the snack they were randomly assigned. For the sugar-free chewing gum group, students chewed gum for 10 minutes and then spit it out. All groups waited for 5 minutes after eating the last bite of food or

after spitting out the gum. The second saliva sample was collected in a different 2 mL tube after this 5 minute waiting period.

A labeling system was created to help maintain the organization of all of the samples collected and to maintain the anonymity of each participant's saliva sample. Names were not associated with codes in this system. "CM" and "CB" were abbreviations of "circadian morning" collection and "circadian bedtime" collection respectively. The four sugar groups each used "S" as the leading letter, followed by a number representing the group: 1= no sugar, 2= low sugar, 3= medium sugar, and 4= high sugar content. This was then followed by another number (either 1 or 2), which represented the sample being either the first or second sample collected. For example, "S21" represents Sugar group 2 (low sugar) and is the first sample for this individual. A final three-digit code was added behind the initial code behind an underscore after each student turned in his or her samples to designate the sample number (001-200). These last three digits went in numerical order as students turned in their samples. These numerical codes were kept constant between an individual's samples, meaning that the same three numbers after the underscore were the same for both samples from the same individual.

The tubes of saliva samples were then frozen for preservation. There is a low likelihood of salivary cortisol being affected before data collection if left alone in the freezer, and the freeze/ thaw cycle is shown to have no affect on salivary cortisol concentration (Kalman et al., 2004; Tennison et al., 2010). The tubes for both the sugar and circadian groups went into separate labeled Ziploc bags before being put in the freezer. Each category had two different bags to separate the first (before eating) from the

second (after eating) samples. The circadian group had one bag labeled “Circadian Bedtime” and another bag labeled “Circadian Morning.”

Three, Eagle Biosciences Salivary Cortisol ELISA Assay kits were used to prepare the saliva samples for analysis in the microplate reader. The steps for the Enzyme Linked Immunosorbent Assay (ELISA) assay included thawing and resuspending each sample with a vortex mixer. Samples were then thawed and randomly placed across three 96 well plates to prepare for analysis. A record of the saliva sample code as it corresponds to its well was kept. 25 µL of saliva were pipetted into each well of a 96 well plate coated with goat anti-rabbit gamma globulin. 25 µL of calibrators (0, 0.1, 0.3, 1.0, 3.0, 10.0 and 30 ng/ml) and 1.0mL each of two control solutions of 1.130 and 0.159 mean absorbance (at 450 nm) was also placed in each well. These wells were then treated with 50 µL of horseradish peroxidase working reagent and 50 µL of antibody. The plates were incubated for one hour at room temperature. The wells were then decanted and washed three times each with diluted wash solution. Each well was treated with 100 µL of color development reagent, which turned the samples blue and incubated for 30 minutes at room temperature. Lastly, 100 µL of stop reagent was added. The samples then turned yellow.

Prepared 96 well plates were analyzed in a BioTek ELx808 Absorbance Microplate Reader to obtain results. Output data for absorbance values were determined for each well with the BioTek Gen5 Microplate Data Collection & Analysis Software. Higher absorbance readings are associated with lower amounts of cortisol being present in that well read at 450nm with the microplate reader. A standard curve was prepared to predict the cortisol concentration values of the unknown samples by using Microsoft

Excel with the known standards of the assay kit and absorbance values from the microplate reader (Fig. 1). The standard curve provided an R^2 value that was used to calculate cortisol concentrations for all samples. A paired T-test compared the night and morning saliva samples. An Analysis of Co-Variance (ANCOVA) was used to obtain results from multiple categories of the sugar treatment groups with the concentration of the initial sample as the covariate. The paired T-test and ANCOVA were conducted using the Statistical Package for the Social Science (SPSS) v22. Significance was set at $\alpha<0.05$ for all tests.

RESULTS AND DISCUSSION

Through the use of a paired t-test, the circadian rhythmicity testing procedure of Liu and Vagula (2015) for cortisol levels was validated. The before bedtime samples for the 16 undergraduate students in the circadian group had a mean cortisol concentration of 1.9883 ng/mL. The morning samples for this same group were significantly higher with a mean cortisol concentration of 6.7803 ng/mL ($t= -5.40$; $df=15$; $p< 0.001$; Fig. 2).

The respective sugar groups of 0g sugar, low sugar (< 1g), medium sugar (14g), and high sugar (26g) content had post-consumption cortisol concentrations that were significantly different between the sugar treatment groups with ($F=8.61$; $df=83$; $p<0.001$; Fig. 3).

The results from the alternate activity of watching a video based on cortisol and endocrine related topics and then taking a quiz on the points discussed within the video were encouraging. 100% of students (n=66) correctly answered the first question, 93.93% the second question, 92.42% for the third, 95.45% for the fourth, and 96.97% answered correctly for the last question.

The physiological response of the HPA Axis is likely induced by stress itself, and may be exacerbated by the sugar-laden foods often eaten in response to stress. Nutrient-poor foods with high sugar content, may also elevate cortisol levels independently from an obvious stressor due to recognition of the formally established reward system associated with stress (Duarte et al., 2014). Relating these unhealthy eating behaviors to cortisol levels, independently of feelings of stress, is of interest for this experiment, which focused on measuring changes in cortisol levels in response to sugar intake. A dip

in cortisol levels is experienced during the afternoon around 3:00, which accompanies the feeling of drowsiness often felt during this time (Adams and Kumari, 2009; Sharpley et al., 2010). There was a significant, 2-fold difference in cortisol concentration between the night and morning saliva samples, which solidifies the robust nature of the circadian rhythm's affect on physiological processes.

The sugar treatment groups differed significantly between samples suggesting that sugar does have some effect in raising cortisol levels. Salivary cortisol concentration did tend to increase incrementally with increasing sugar, with the exception of the medium sugar group having higher salivary cortisol levels than the high sugar group. This could suggest that amount of sugar does not influence cortisol as much as the actual presence of sugar intake. Even smaller amounts of sugar may act as a stressor, causing the HPA axis to release more cortisol into the bloodstream (Haussmann et al., 2006).

High sugar consumption has been associated with increased visceral adipose tissue, and high sugar intake may influence the role between cortisol and adipose tissue, which could be of interest in obesity studies (Gyllenhammer et al., 2014). Overweight minority adolescents were measured for height, weight, fat composition, etc., and stayed at a research facility overnight for cortisol measurements and assessment of their dietary intake (Gyllenhammer et al., 2014). These minority youth tended to weigh more than their peers, and they also developed more weight related diseases (Gyllenhammer et al., 2014). It was deduced that high sugar intake likely influences the role between cortisol and adipose tissue (Gyllenhammer et al., 2014). My hypothesis that sugar intake increases cortisol was upheld and supports this relationship established by Gyllenhammer et al. (2014). This provides another level of complexity to the relationship between sugar,

cortisol, and obesity, which would be of interest in a future study. Exploring further ties between sugar intake as a reward for taking challenging college courses and struggling with time management in undergraduate students would be of interest for comparing weight before and after the university experience. This information could be used to further encourage schools and universities to adopt more healthy alternatives to the sugar-laden foods and beverages characteristic of a college campus.

Some limitations of the undergraduate salivary cortisol experiment include extraneous circumstances that may have resulted in relying on students to provide their own samples especially those in the circadian group who collected their samples and froze them at home and then brought them in the next day. No specific bedtime was set, so the times of sleeping and waking differed among individuals. Cortisol levels in women have been shown to be affected by birth control and the menstrual cycle (Gozansky et al., 2005; Ormsbee et al., 2013). A woman taking oral contraceptives showed abnormal results in a serum test for cortisol (Gozansky et al., 2005). In the future, separate data for males and females should be kept to note any differences and to account for any differences based on gender.

First generation college students tend to have higher cortisol levels due to a typically more difficult adjustment period (Stephens et al., 2012). Differences in stress level related to anxiety may relate to higher concentrations of cortisol being released as part of the stress cascade for these students. It would be of interest to mentors, counselors, or parents of these children to encourage them to practice self-care techniques to relieve these feelings of anxiety. Mindfulness techniques like meditation have been shown to aid

in lowering cortisol levels (Fan et al., 2013; Ramler et al., 2015). Aromatherapy is another soothing technique shown to combat feelings of stress with lavender, ylang-ylang, marjoram, neroli, clary sage oil, and orange essential oil being good options to use for this technique (Jaafarzadeh et al., 2012; Kim et al., 2015; Lee et al., 2014). Integrative body-mind training (IBMT) combines meditation, mindfulness, and other relaxation techniques to reduce stress in an even more effective way than common mindfulness techniques (Fan et al., 2013). Students could be given some information on these techniques during the lab to provide tools for how to help lessen the anxiety and loneliness felt by many of them in the university setting (Ramler et al., 2015; Sladek and Doane, 2014; Stephens et al., 2012).

There is a link between glucose and lipid metabolism, cortisol, melatonin, and other hormones with the circadian rhythm that has been shown to be negatively impacted by disruptions in the sleep pattern such as in changing shifts at work (Kim et al. 2015). The importance of sleep for hormonal and metabolic function could also be presented to students so they avoid some of the pitfalls of unhealthy habits of college students. Obesity, diabetes, and insulin insensitivity are just some of the known potential drawbacks caused by the circadian dysfunction that results in hormonal imbalances supported by the increase in cortisol due to sugar consumption seen in this experiment (Gyllenhammer et al., 2014; Kim et al. 2015). Many students may be aware of a general importance for proper sleep and nutrition; however, this laboratory exercise would provide them with a deeper understanding of the physiological reasons why these things are said to be important.

The quiz based on the content of a video in which a doctor discussed the circadian rhythm and cortisol among other related topics seemed to be another useful aid in learning the material for the topic of interest of endocrine processes. It could be provided in addition to the lab exercise as supplemental material. The video selected seemed to be effectively matched with appropriate quiz questions, as the majority of students were able to answer the quiz questions correctly. As expected, generic questions that were repeatedly mentioned in the video such as the expected time of day for lower versus higher levels of cortisol were answered correctly by a higher percentage of students. More specific questions that were only mentioned one or two times in the video tended to have a slightly lower percentage of students who were able to answer them correctly.

Salivary cortisol experiments are an effective way to involve elements of the endocrine system in a university laboratory setting. The average biology laboratory time for undergraduates at the University of Mississippi is limited to around 2-3 hours per week. Hormones of the endocrine system tend to be slower to assess than elements of other systems such as the rapid responses characteristic of the nervous system. Some logistical limitations include the cost for students to do the activity, preparation of the 96 well plates, and planning what to do while the students wait during the incubation period of the samples. Developing an exercise that limits the time spent on collection methods and maximizes analysis of results and clarifying the information of interest should be considered in the development of a laboratory exercise for undergraduate students. Limiting resources and steps involved to complete the exercise should also be considered to limit potential areas of confusion for students.

This experiment could potentially be integrated into the laboratory curriculum as part of the endocrine system learning material for the Introductory Physiology class at the University of Mississippi. The data collection process was able to be completed in around 30 minutes and the analysis in 2 hours, which is a reasonable amount of time for a standard lab period of 3 hours, especially if the students work together in groups. This would provide an interactive endocrine-based laboratory exercise to help aid in learning the material taught in class with an additional focus on representing the effect of sugar intake on some physiological processes. This exercise benefits students by allowing them to eat food, which many of them will likely find enjoyable. This experiment seems to be a success in its ability to be integrated into an undergraduate lab curriculum due to its relevance to the subject matter taught in class, the accessibility of saliva, and its overall feasibility.

LIST OF REFERENCES

Crewther, B.T., Lowe, T.E., Ingram, J., & Weatherby, R.P. (2010) Validating the Salivary Testosterone and Cortisol Concentration Measures in Response to Short High-intensity Exercise. The Journal of Sports Medicine and Physical Fitness, 50(1), 85-92

Duarte, R.B.M, Patrono, E., Borges, A.C., Tomaz, C.Ventura, R, Gasbarri, A., Puglisi-Allegra, S, & Barros, M. (2014). High Versus Low Fat/sugar Food Affects the Behavioral, but not the Cortisol Response of Marmoset Monkeys in a Conditioned-place-preference Task. Physiology and Behavior. 139, 442-448

Fan, Y., Tang, Y.Y., & Posner, M.I. (2013) Cortisol Level Modulated by Integrative Meditation in a Dose-dependent Fashion. Stress & Health: Journal of the International Society for the Investigation of Stress, 30, 65-70

Gozansky, W.S., Lynn, J.S., Laudenslager, M.S., & Kohrt, W.M. (2005) Salivary Cortisol Determined by Enzyme Immunoassay is Preferable to Serum Total Cortisol for Assessment of Dynamic Hypothalamic–pituitary–adrenal axis Activity. Clinical Endocrinology, 63, 336-341

Gyllenhammer, L.E., Weigensberg, M.J., Spruijt-Metz. D., Allayee, H., Goran, M.I., &

Davis, J.N. (2014) Modifying Influence of Dietary Sugar in the Relationship Between Cortisol and Visceral Adipose Tissue in Minority Youth. Obesity, 22, 474-481

Haussmann, M.F., Vleck, C.M., & Farrar, E.S. (2006) A Laboratory Exercise to Illustrate Increased Salivary Cortisol in Response to Three Stressful Conditions Using Competitive ELISA. Advances in Physiology Education, 31, 110-115

Jaafarzadeh, M., Arman, S., & Pour, F.F. (2012) Effect of Aromatherapy with Orange Essential Oil on Salivary Cortisol and Pulse Rate in Children during Dental Treatment: A Randomized Controlled Clinical Trial. Advanced Biomedical Research, 2(1), 1-7

Kalman, B.A. & Grahn, R.E. (2004). Measuring Salivary Cortisol in the Behavioral Neuroscience Laboratory. Journal for Undergraduate Neuroscience Education, 2(2), A41-A49. http://www.funjournal.org/

Kim, I.N., Kim, C., Seong, K., Hur, M.H., Lim, H.M., & Lee, M.S. (2012) Essential Oil Inhalation on Blood Pressure and Salivary Cortisol Levels in Prehypertensive and Hypertensive Subjects. Evidence-Based Complementary and Alternative Medicine, 2012, 1-9

Kim, T.W., Jeong, J.H., & Hong, S.C. (2015) The Impact of Sleep and Circadian

Disturbance on Hormones and Metabolism. International Journal of Endocrinology, 2015, 1-9

Kumari, M. & Adam, E.K. (2009) Assessing Salivary Cortisol in Large-scale, Epidemiological Research. Psychoneuroendocrinology, 34, 1423-1436

Lee, K.B., Cho, E., & Kang, Y.S. (2014) Changes in 5-hydroxytryptamine and Cortisol Plasma Levels in Menopausal Women After Inhalation of Clary Sage Oil. Phytotherapy Research, 28, 1599-1605

Liu, H. & Vagula, M.C. (2015). An Undergraduate Physiology Laboratory Module of Salivary Cortisol Measurement with an Emphasis on Circadian Rhythmicity and Quantitative Analysis. Journal of the Human Anatomy and Physiology Society, 19(3), 22-25.

Ormsbee, M.J., Kinsey, A.W., Chong, M., Friedman, H.S., Dodg, T., & Fehling, P.C. (2013). The Influence of High Intensity Interval Training on the Salivary Cortisol Response to a Psychological Stressor and Mood State in Non-Sedentary College Students. Journal of Exercise Physiology Online. 16(1), 105-116

Ramler, T.R., Tennison, L.R., Lynch, J., & Murphy, P. (2015). Mindfulness and the

College Transition: The Efficacy of an Adapted Mindfulness-Based Stress Reduction Intervention in Fostering Adjustment among First-Year Students. Mindfulness, 7, 179-188

Sharpley, C.F., Kauter, K.G., & McFarlane, J.R. (2010) Diurnal Variation in Peripheral (Hair) vs. Central (saliva) HPA Axis Cortisol Concentrations. Clinical Medicine Insights: Endocrinology and Diabetes, 3, 9-16

Sladek, M.R. & Doane (2014) Daily Diary Reports of Social Connection, Objective Sleep, and the Cortisol Awakening Response During Adolescents' First Year of College. Journal of Youth and Adolescence, 44, 298-316

Stephens, N.M., Townsend, S.S.M, Markus, H.R., Philips, L.T. (2012). A Cultural Mismatch: Independent Cultural Norms Produce Greater Increases in Cortisol and More Negative Emotions among First-generation College Students. Journal of Experimental Social Psychology, 48, 1389-1393

Stowell, J.R., Tumminaro, T., & Attarwala, M. (2008) Moderating Effects of Coping on the Relationship between Test Anxiety and Negative Mood. Stress and Health, 24, 313-321

Tennison, L.R., Rogers, L.S., Berker, D., Vorobjeva, K.I., Creed, E.T., & Simonenko, A.

(2010) Cortisol and Symptoms of Psychopathology in Russian and American College Students. International Journal of Psychology, 5 (3), 165–173

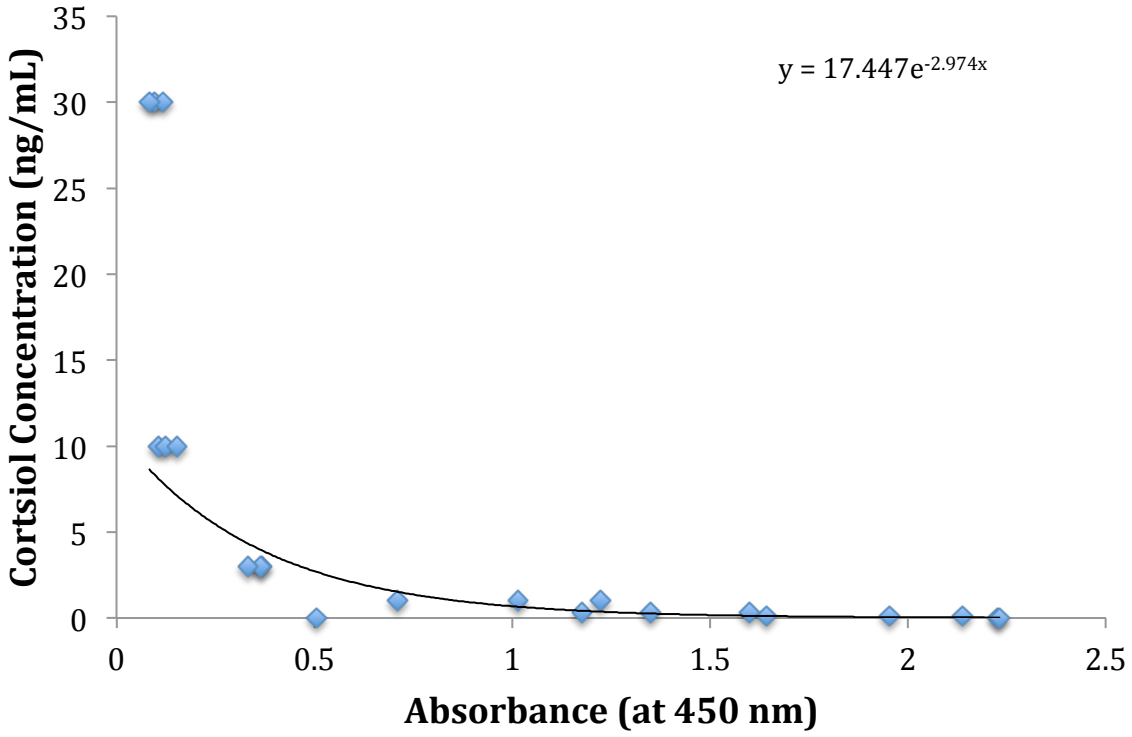

Figure 1: Standard curve of absorbance values with known standards of (0, 0.1, 0.3, 1.0, 3.0, 10.0) and 30 ng/ml) from the manufacturer, Eagle Biosciences is used to create the equation $y = 17.447e^{-2.974x}$ which was used to calculate concentrations of cortisol for all samples.

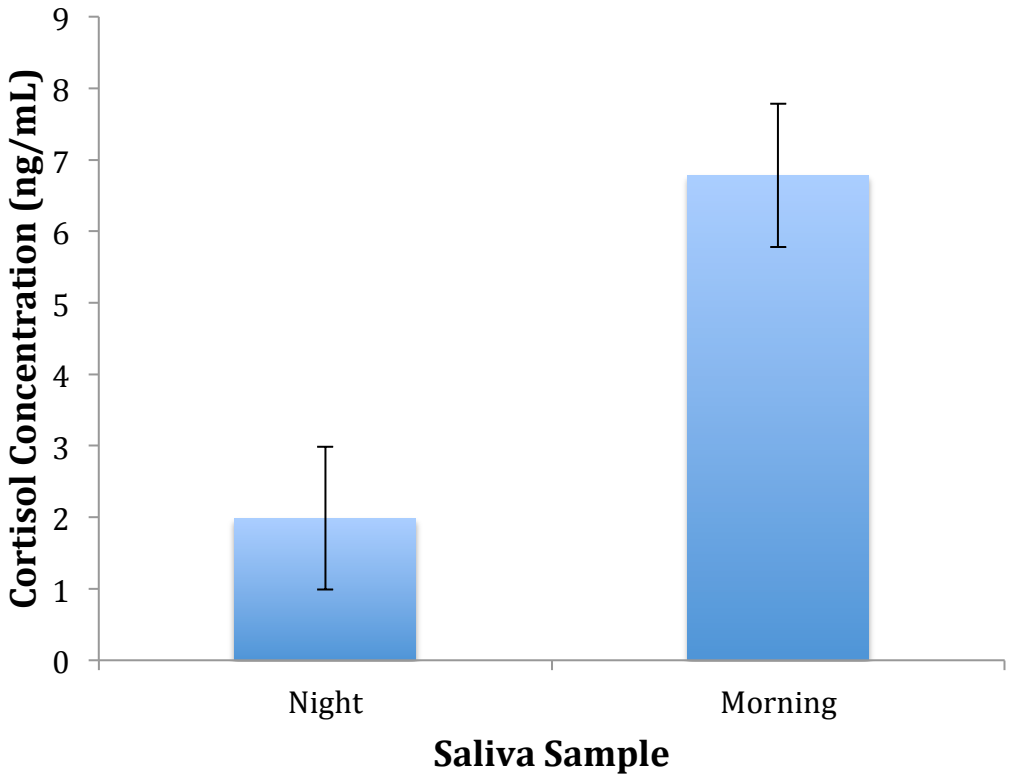

Figure 2: Salivary cortisol concentration of a group of undergraduate students (n=16) was found to be significantly higher in the upon waking morning sample, than in the saliva sample collected at night before sleeping ($t=-5.402$; $df=15$; $p<0.001$).

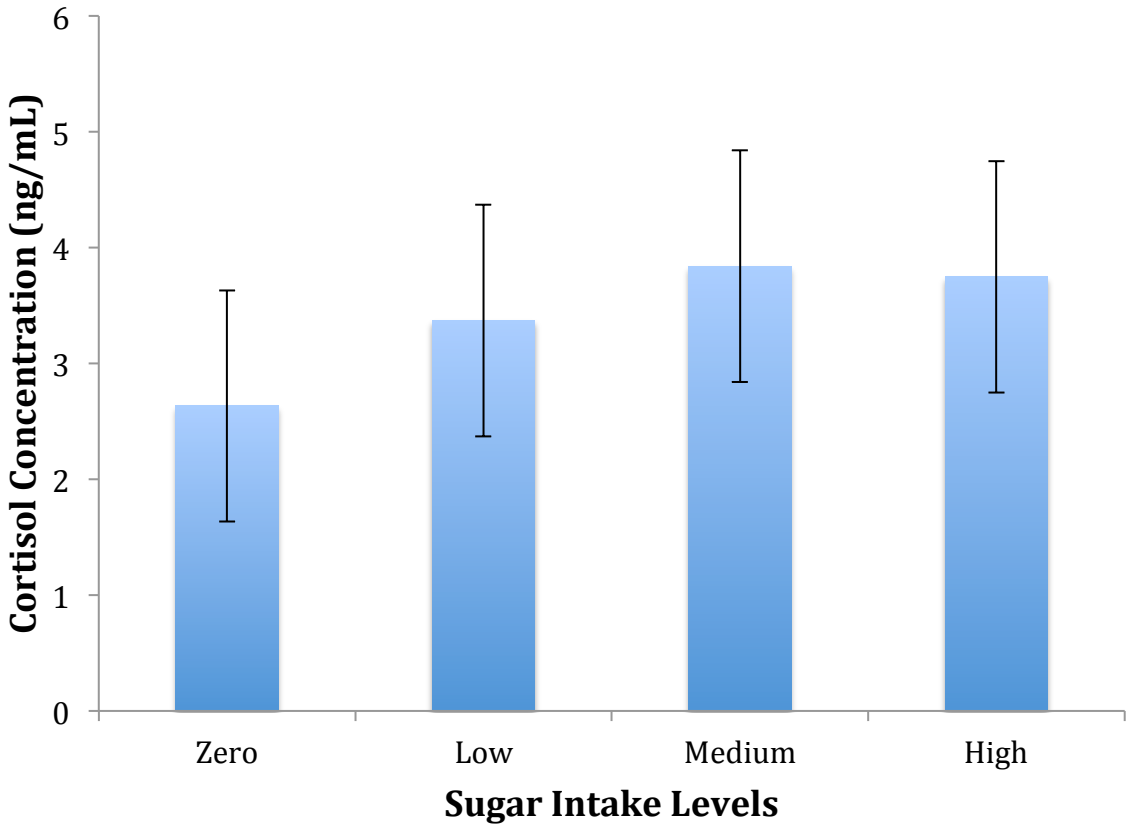

Figure 3: Salivary cortisol concentration of undergraduate students after eating certain snacks are shown for the 0g sugar consumption group (n=19), low sugar consumption group (n=20), medium sugar consumption group (n=20), and high sugar consumption group (n=25) with standard deviation. An ANCOVA showed that post-consumption cortisol levels differed significantly between subjects of the treatment groups ($F=8.61$; $df= 83$; $p<0.001$).

APPENDIX A

Alternative Quiz for Human A&P Students

Video on YouTube (10 minutes)

https://www.youtube.com/watch?v=Lkb-CZRON0E

Questions:

1.Are cortisol levels in the body usually higher at night or in the morning?

A) Morning

B) Night

2.Which disease discussed in this video results in low cortisol levels?

A) Diabetes

B) Cushing's Syndrome

C) Addison's Disease

D) AIDS

3. Which method of cortisol testing is a more accurate representation of cellular functioning?

A) Blood testing

B) Skin Sampling

C) Urine Test

D) Saliva Test

4. Which example is repeatedly used in the video to represent a cause for a large spike in cortisol levels?

A) Being chased by a bear

B) Car Crash

C) Classwork

D) A Robbery

5. A sign of being stuck in a “stress mode” is…

A) Grinding Teeth

B) Biting Fingernails

C) Raised Shoulders

D) All of the Above

Answers: 1)A 2)C 3)D 4)A 5)D